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EFFECT OF TEMPERATURE ON PARTITION BEHAVIOUR AND THERMODYNAMIC CHARACTERISTICS OF BIOACTIVE PHENOLICS TRANSFER IN AQUEOUS TWO-PHASE EXTRACTION SYSTEM AND EVALUATION OF ANTIMICROBIAL ACTIVITY

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ABSTRACT

The present study was to develop a cost-effective and eco-friendly separation and purification method of bioactive phenolics from *Phanerochaete chrysosporium* biomass using aqueous two-phase extraction system. The extraction system comprised of 50% (w/w) ethanol, 20% (w/w) di-potassium hydrogen phosphate, distilled water and solid biomass. The extraction was conducted at different temperatures range, from 25°C to 65°C for 2 hours at pH 7. The highest partition coefficient (K) and recovery (R) of bioactive phenolics were found at 25°C with values of 2.37 and 93.89%, respectively. From the thermodynamics analysis, the Gibbs energy change (ΔG), enthalpy change (ΔH) and entropy change (ΔS) values were -2138.95kJmol^{-1}, -11504.91 kJmol^{-1} and-31.10 kJmol^{-1}, respectively. These negative values reflected that the extraction process was spontaneous, exothermic and preferential partitioning in the ethanol-rich phase at lower extraction temperature. Antimicrobial activities were assayed using well and diffusion methods. The extract at 25°C showed the highest zone of inhibition against *Aspergillus niger*and *Escherichia coli*.Finally, the bioactive phenolic constituents were analyzed using reversed-phase high performance liquid chromatography (RP-HPLC).

KEYWORDS: Aqueous Two-Phase Extraction, Phenolics, Partition Behaviour, Thermodynamic Characteristics, Antimicrobial Activity

INTRODUCTION

Phenolic compounds are known bioactive constituents from plants and basidiomycetes that have remarkable potential forretarding the growth of various spoilage and pathogenic microorganisms. Their activity against human pathogens has been investigated to characterize and develop new functional food, medical and pharmaceuticals [1].

Phanerochaete chrysosporium is a filamentous basidiomycete white rot fungus that participates in the degradation process of complex woody materials. This fungus has great potential in many biotechnological applications including bioprotein production, the treatment of hazardous waste and the bioremediation of contaminated soils and biofuel

production [2]. Owing to the increasing interest in new natural sources of antimicrobial compounds, this is the first study on *Phanerochaete chrysosporium* mycelia biomass as potential source of bioactive compounds.

Extraction is the most important step in the recovery and purification of bioactive compounds from plants and basidiomycetes. Phenolic compounds are usually extracted from natural sources through solid-liquid extraction using organic solvents in heat-reflux systems [3].However, this method has suffered low efficiency, time and solvent consuming, could lead to the degradation of bioactive phenolics and decrease the bioactivity of the extracts.

Aqueous two-phase extraction is recognized as an effective, versatile and important emerging technique for recovery and purification of biomolecules. It will achieve high product purity as well as high yield, while maintaining the biological activity of the molecule, which has been widely applied in the separation of proteins, enzymes, antibiotics and polyphenols [4].

In the present study, the effect of temperature on the partitioning of the bioactive phenolics from *Phanerochaete chrysosporium* mycelia biomass and antimicrobial activity of the extracts were investigated in order to develop an efficient aqueous two-phase system for the extraction and purification of these compounds.

MATERIALS AND METHODS

Chemicals and Reagents

Folin-Ciocalteu reagent standards, and other solvents and reagent of analytical grade were purchased from Merck Chemicals (Darmstadt, Germany).

Fungal Strain and Preparation of Inoculum

The white rot fungus *Phanerochaete chrysosporium* was obtained from the culture collection of School of Bioprocess Engineering, Universiti Malaysia Perlis. Four square plugs (5 mm in length) of active mycelia were cut from the initial culture plate and transferred on new potato dextrose agar (PDA) plate. The plate was incubated at 30°C for 5 days. The inoculum was prepared by washing four PDA plates cultured with 100 ml of sterile water and their spore suspension were rubbed and poured into 250 ml Erlenmeyer flask.

Submerged Fermentation

The fermentation was done at fixed media compositions. The substrate used in the study was 1% (v/v) of diluted molasses (as carbon source). The media was supplemented with 0.2% (w/v) of potassium dihydrogen phosphate (KH_2PO_4), 0.2 % (w/v) of manganese sulfate ($MnSO_4$) and 1% (w/v) of ammonium nitrate (NH_4NO_3) to enhance the strain's growth. Fifty millilitres of sample were taken into 100-ml Erlenmeyer flasks and autoclaved at 121°C for 30 min. The sterile media was cooled to ambient temperature and inoculated with spore suspension of *Phanerochaete chrysosporium*. The fermentation was carried out at incubation time of 3 days, 4% (v/v) of inoculum size, inoculum age of 7 days, pH 6, agitation speed of 100 rpm, and fermentation temperature of 25°C. For reproducibility of results, all fermentations were carried out in triplicate.

Preparation of Mycelia Biomass Powder

After fermentation, the mycelia biomass of *Phanerochaete chrysosporium* was collected by filtration using filter paper and rinsed with distilled water. The biomass was dried in oven at 60°C for 24 h and ground into powder by using mortar and pestle.

Aqueous Two-Phase Extraction

The phase system was prepared in a 15ml graduated centrifuge tubes by weighing the appropriate amount use which is 50 wt % of ethanol, 15wt % of potassium salts and 0.028g of sample powder. Distilled water was added to the system to obtain final mass of 14g. The phase system was mixed evenly by gentle agitation, after that each phase system was centrifuged at 3000×g for 10minutes to induce phase separation. The phase diagram used was developed using cloud point method. The extraction was conducted at different temperatures range, from 25°C to 65°C for 2 hours at pH 7. The partition coefficient (K) and the percentage of recovery, R (%) of bioactive phenolics were calculated as follows:

$$K = \frac{C_T}{C_B} \tag{1}$$

$$R_T(\%) = \frac{100 C_T V_T}{(C_T V_T + C_B V_B)} \tag{2}$$

$$R_B(\%) = \frac{100 C_B V_B}{(C_T V_T + C_B V_B)} \tag{3}$$

C_T and C_B represents the concentrations of bioactive phenolics in the top and bottom phases, respectively. V_T and V_B are the volumes of the top and bottom phases, respectively.

Thermodynamic Parameters of Bioactive Phenolics Transfer

The thermodynamic parameters of transfer of bioactive phenolics in aqueous two-phase system were determined by Van't Hoff approach by plotting the ln (K) versus T^{-1} which will exhibit linear relationship given by the following equations [5]:

$$\ln(K) = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \tag{4}$$

$$\Delta G = -RT \ln(K) \tag{5}$$

K is the partition coefficient of bioactive phenolics, R is the universal gas constant (8.314 J mol^{-1} K^{-1}), T is the temperature (K), and ΔH, ΔS and ΔG are the enthalpy change of transfer, the entropy change of transfer and the Gibbs energy change of bioactive phenolics transfer, respectively.

Determination of Total Phenolic Content

The total phenolic content was determined based on the suggested method with modification [2], using the Folin-Ciocalteu reagent with gallic acid as a standard. In 15 ml test tube, 2.37 ml of distilled water, 0.03 ml of sample extract or blank and 0.15 ml of Folin-Ciocalteu reagent were added and vortexed. After 1 min, 0.45 ml of 20% saturated sodium carbonate (Na_2CO_3) was added, and then the mixture was vortexed and allowed to stand at 40°C for 30 min. The absorbance was taken at 750 nm. The total phenolic content was expressed as mg of gallic acid equivalent per liter (GAE mg/l). All measurements were measured in triplicate.

Determination of Antimicrobial Activity

The antimicrobial study was conducted for the determination of zone inhibition. The phenolic extracts were tested for antimicrobial activity by disc diffusion method [6]. This method of antimicrobial activity was evaluated by measuring

the inhibition zones that was recorded as the diameter of growth free zone including the diameter of the well (in millimeters) after the incubation period. The phenolic extracts were classified as active when the diameter of the inhibition zone was equal to a larger than 6 mm. A negative control of sterile distilled water was included as the negative control.

HPLC Analysis

The HPLC method was conducted on a Shimadzu liquid chromatograph system (Shimadzu Corp, Kyoto, Japan) equipped with a quaternary pump, a vacuum degasser, an autosampler, a UV-vis detector, and a Syncronis^{TM} C18 column (250 mm × 4.6 mm, 5 µm). Results were acquired and processed by the Shimadzu Workstation CLASS-VP 6.12 software. Mobile phase consisted of 2% (v/v) acetic acid in water-methanol 82:18 (v/v), flow rate 1.2 ml/min Phenolic acids were detected at a wavelength of 280 nm [7].

RESULTS AND DISCUSSIONS

Partition Behaviour of Bioactive Phenolics in Aqueous Two-Phase Extraction System

The extraction of bioactive phenolics was conducted at different temperatures range of 25°C to 65°C for 2 hours at pH 7 to investigate their effect on the separation of bioactive phenolics from *Phanerochaete chrysosporium* biomass using aqueous two-phase extraction system. The optimum extraction system comprised of 50% (w/w) ethanol, 20% (w/w) di-potassium hydrogen phosphate and distilled water was selected from the phase diagram of ethanol with K_2HPO_4 at 25°C as shown in Figure 1.

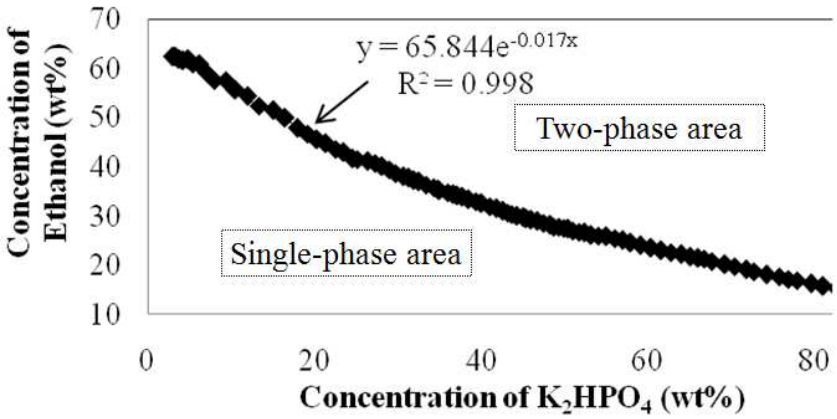

Figure 1: Phase Diagram of Ethanol + K_2HPO_4 + H_2O at 298.15 K

Higher temperature causes the increase of salt solubility into water which leads to faster phase-separation [8]. In this study however, both values of K and R of the bioactive phenolics decreased as the temperature increased as shown in Figure 2. Higher temperature also causes the degradation of bioactive phenolics which may be responsible for the slight decrease of K and R when the temperature is higher than 25°C. From the results, the highest partition coefficient (K) and recovery (R) of bioactive phenolics to the ethanol-rich phase were found at 25°C with values of 2.37 and 93.89%, respectively.

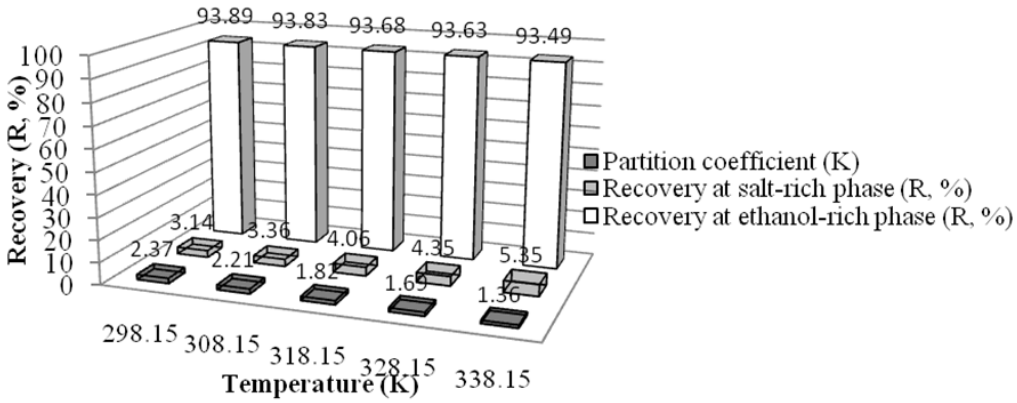

Figure 2: Effect of Extraction Temperature on the Partition Coefficient (K) and Recovery (R) of Bioactive Phenolics in Ethanol/K_2HPO_4 Based System

Thermodynamic Characteristics of Bioactive Phenolics Transfer

For analysis of thermodynamic properties, Van't Hoff plot of K versus 1/T [7] was constructed as shown in Figure 3. From the graph, a linear correlation coefficient (R^2) was 0.9609, indicating a good linearity over the investigated ranges. The linear regression analysis was used to determine the Gibbs energy change (ΔG), enthalpy change (ΔH), and entropy change (ΔS) of the extraction system.

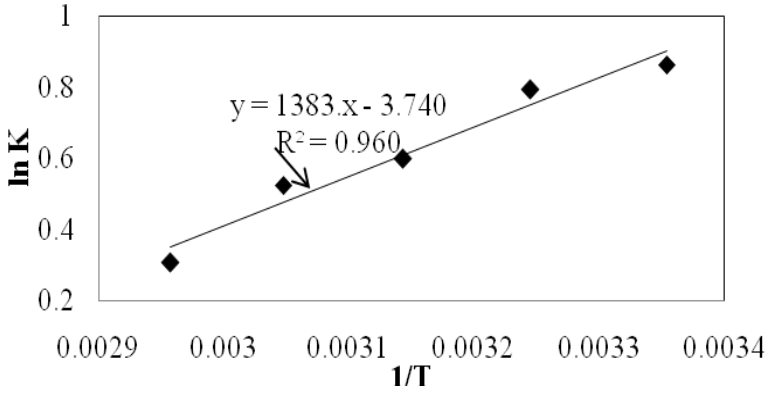

Figure 3: The Graph of ln K versus 1/T

The ΔH and ΔSwere obtained from the slope and intercept of the linear equation, y = 1383.8x – 3.7406 which gave the values of -11504.91 kJ mol^{-1} and -31.10 kJ mol^{-1}, respectively. The ΔG was determined using Equation 5 and tabulated in Table 1.From this thermodynamic analysis, all the negative values of ΔG, ΔH and ΔS reflected that the extraction process was spontaneous, exothermic and preferential partitioning in the ethanol-rich phase at lower extraction temperature.

Table 1: Thermodynamic Parameters of Bioactive Phenolics Transfer

Temperature (K)	K	ΔG(kJ mol^{-1})	TΔS (kJ mol^{-1})	ΔH(kJ mol^{-1})
298.15	2.37	-2138.95	-9272.27	-11504.91
308.15	2.21	-2031.61	-9583.26	
318.15	1.82	-1583.98	-9894.26	
328.15	1.69	-1431.58	-10205.25	
338.15	1.36	-864.456	-10516.24	

Evaluation of Antimicrobial Activity

The effect of extraction temperature on antimicrobial activity of the extracts against *Escherichia coli* and *Aspergillus niger* was investigated. The results were shown in Table 2 and it was observed that with the increase of extraction temperature, the inhibition zone diameter decreased for both microorganisms. The extracts showed the highest zone of inhibition against *Escherichia coli* (11.33±0.58 mm) and *Aspergillus niger* (25.00±0.82 mm) at lower extraction temperature of 25°C. The decrease of the antimicrobial activity of the extracts against extraction temperature was significantly correlated with the values of K and R of the bioactive phenolics obtained in previous result. Higher temperature causes the degradation of bioactive phenolics thus reduced the antimicrobial activity of the extracts.

Table 2: Inhibition Zone Diameter (Mm) of Extracts at Different Extraction Temperatures

Temperature (°C)	*Escherichia coli*	*Aspergillus niger*
25	11.33±0.58	25.00±0.82
35	10.50±0.50	23.00±0.82
45	9.50±0.50	21.24±1.26
55	8.50±0.50	19.50±0.58
65	8.33±0.29	18.63±0.48

HPLC Analysis

The HPLC profile of *Phanerochaete chrysosporium* biomass extracted at 25°C is shown in Figure 4. Out of four standards, only gallic acid was matched. Nochlorogenic 3,4-dihydroxybenzoic and 4-hydroxybenzoic acids were detected in the extracts. The same profile was also observed for the extracts at 35°C, 45°C, 55°C and 65°C.

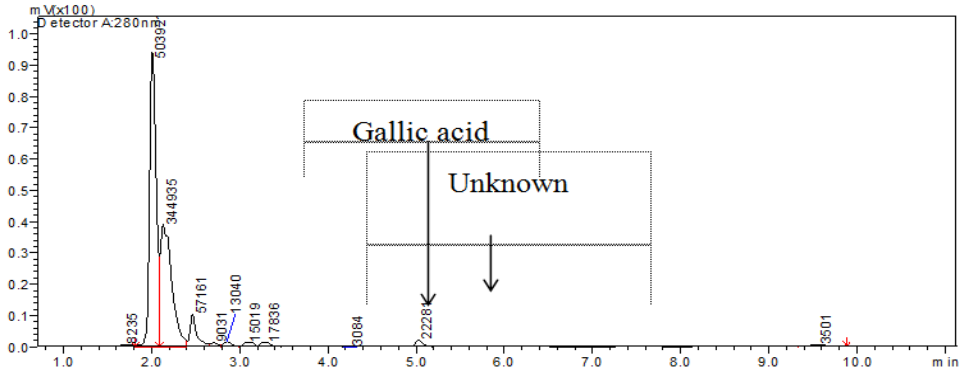

Figure 4: HPLC Chromatogram of *Phanerochaete chrysosporium* Biomass Extract at 25°C

The distribution of gallic acid in extracts at different extraction temperatures was shown in Table 3. It was observed that there was no significant difference between the concentrations of gallic acid against the extraction temperature. The gallic acid concentration was found in the range of 0.0621 to 0.0624 mg/ml

Table 3: Phenolic Acid Concentration (mg/ml) in the Extracts at Different Extraction Temperatures

Temperature (°C)	Chlorogenic Acid	3,4-Dihydroxybenzoic Acid	Gallic Acid	4-Hydroxybenzoic Acid
25	n.d	n.d	0.0622	n.d
35	n.d	n.d	0.0623	n.d
45	n.d	n.d	0.0624	n.d
55	n.d	n.d	0.0621	n.d
65	n.d	n.d	0.0624	n.d

From the HPLC results, the present of gallic acid might responsible for the antimicrobial activity of the extracts. Gallic acid was reported to have high antimicrobial activity against several types of Gram-negative bacteria including *Escherichia coli* [9]. However, gallic acid was not the only compound that showed antimicrobial activity of the extracts. This due to fact that the gallic acid constituent was almost equally distributed in each of the extract. The unknown peak with retention time of 4.8 to 5.1 min was also detected in the HPLC profile. The area under the curve of this unknown compound was quite larger as compared to the gallic acid and it showed positive correlation with the partition coefficient (K), the recovery (R) and the antimicrobial activity.

CONCLUSIONS

This study demonstrated that the aqueous two-phase extraction system consisted of 50% (w/w) ethanol, 20% (w/w) di-potassium hydrogen phosphate and distilled water was successfully separated and purified bioactive phenolics from *Phanerochaete chrysosporium* biomass. The highest partition coefficient (K) and recovery (R) of bioactive phenolics were found at 25°C with values of 2.37 and 93.89%, respectively. From the thermodynamics analysis, the Gibbs energy change (ΔG), enthalpy change (ΔH) and entropy change (ΔS) values were -2138.95kJmol^{-1}, -11504.91 kJmol^{-1} and-31.10 kJmol^{-1}, respectively. These negative values reflected that the extraction process was spontaneous, exothermic and preferential partitioning in the ethanol-rich phase at lower extraction temperature. The extract at 25°C showed the highest zone of inhibition against *Aspergillus niger* and *Escherichia coli*. Gallic acid was identified with an unknown compound which might responsible for the antimicrobial activity. For further study, the identity of the unknown compound will be investigated.

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